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13. ABSTRACT ***(Maximum 200 Words)*** Angiopoietin-1 (Ang-1) and its receptor Tie-2, a trans-membrane tyrosine kinase uniquely expressed by endothelial cells, are essential to developmental angiogenesis. The phenotypic abnormalities shown by null mutation studies suggest that Tie-2 signaling is necessary for the maintenance and expansion of the primitive capillary network. We present in vitro evidence indicating that the Ang-1/Tie-2 system participates in the regulation of capillary tubule formation and is necessary for the survival of confluent endothelial cells. Although recombinant Ang-1, which induces Tie-2 phosphorylation, has no effect on the proliferation of endothelial cells, treatment of confluent ABAE cells grown on collagen gels with Ang-1 (100 ng/ml) causes the cells to migrate into the collagen gel and form capillary-like tubules. A soluble form of the Tie-2 extra-cellular domain blocks Ang-1 induced tubule-formation. Specific elimination of Tie-2 protein expression in cultured adult bovine aortic endothelial cells (ABAE) as a result of transfection with an antisense oligonucleotide causes cell death in a dose dependent manner (IC50 = 50 nM), with a 6-fold increase in the rate of apoptosis. These findings are consistent with the view that Ang-1/Tie-2 signaling is essential for both angiogenesis and endothelial cell survival.

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INTRODUCTION

The goal of the proposed research is to investigate the possibility of using Tie-2, an endothelial cell-specific receptor tyrosine kinase, as a therapeutic target. Angiopoietin-1 (Ang-1) and its receptor Tie-2, a trans-membrane tyrosine kinase uniquely expressed by endothelial cells, are essential to developmental angiogenesis. The phenotypic abnormalities shown by null mutation studies suggest that Tie-2 signaling is necessary for the maintenance and expansion of the primitive capillary network. We present *in vitro* evidence indicating that the Ang-1/Tie-2 system participates in the regulation of capillary tubule formation and is necessary for the survival of confluent endothelial cells. Although recombinant Ang-1, which induces Tie-2 phosphorylation, has no effect on the proliferation of endothelial cells, treatment of confluent ABAE cells grown on collagen gels with Ang-1 (100 ng/ml) causes the cells to migrate into the collagen gel and form capillary-like tubules. A soluble form of the Tie-2 extra-cellular domain blocks Ang-1 induced tubule-formation. Specific elimination of Tie-2 protein expression in cultured adult bovine aortic endothelial cells (ABAE) as a result of transfection with an antisense oligonucleotide causes cell death in a dose dependent manner (IC_{50} = 50 nM), with a 6-fold increase in the rate of apoptosis. These findings are consistent with the view that Ang-1/Tie-2 signaling is essential for both angiogenesis and endothelial cell survival.

BODY

Background

Endothelial cell organization into functioning vessels is an essential early developmental process and is central to pathological processes such as tumor formation. Endothelial cell proliferation and organization is under regulation, at least in part, of ligands signaling through endothelial cell specific, trans-membrane receptor tyrosine kinases, in particular the vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and 2) (Fong *et al.* 1995; Shalaby *et al.* 1995) and the Tie receptors (Tie-1and Tie-2) (Dumont *et al.* 1994; Sato *et al.* 1995). The ephrin family of factors may be responsible for further differentiation of the arterial and venous systems (Wong *et al.* 1997). Studies in transgenic animals deficient for VEGFR 1and 2 indicate that VEGF signaling is crucial to the initial expansion of the endothelial cell population and its formation into capillary tubules (Fong *et al.* 1995; Shalaby *et al.* 1995). VEGFR-1 and VEGFR-2 (also known as Flk-1 and KDR) (Terman *et al.* 1992; Quinn *et al.* 1993) are endothelial cell specific and are also up regulated in pathological angiogenesis (Ferrara 1995), whereas VEGF-R3 may have non endothelial cell functions (Soker *et al.* 1998).

The phenotypes of transgenic mice deficient for Tie-1 and Tie-2 indicate functions for these receptors in the organization of the expanding capillary network (Dumont *et al.* 1994; Sato *et al.* 1995).Two ligands have been identified for Tie-2 which are called angiopoietin-1 and angiopoietin-2 (Ang-1 and Ang-2). The former activates the receptor and the latter antagonizes this effect by competitive inhibition at the receptor-binding (Davis *et al.* 1996; Maisonpierre *et al.* 1997). No ligand for Tie-1 has been reported to date. The binding of Ang-1 to Tie-2 results in receptor auto-phosphorylation, and subsequent binding to the downstream proteins GRB-2 and SH-PTP2, perhaps after receptor dimerization (Huang *et al.* 1995). GRB-2 and SH-PTP2 are involved in signaling pathways that can result in alteration in cellular morphology and differentiation.

Tie-2 and Ang-1 knockout mice have a lethal phenotype *in utero* at day E10.5 and day E12.5 respectively. (Dumont *et al.* 1994; Sato *et al.* 1995; Suri *et al.* 1996). Tie-2 is not expressed in the embryo until day E8.5, after the expression of the VEGF receptors (Dumont *et al.*, 1995), indicating that expression of Tie-2 may not be necessary for the initial expansion of the endothelial cell lineage but may be necessary for its later remodeling and maintenance. Dumont *et al* (1994) suggest that absence of Tie-2 signaling at this later stage in the development of the circulatory system results in selective endothelial cell apoptosis that is responsible for the lethal phenotype of these transgenic animals. *In vitro* studies indicate that binding of Ang-1 to Tie-2 also results in binding of phosphatidyl-inositol 3 kinase and subsequent activation of protein kinase B/Akt, further supporting the hypothesis that Tie-2 signaling is anti-apoptotic (Kontos *et al.*, 1998).

Morphological analysis of the Tie-2 and Ang-1 knockout animals also indicates a dynamic role in angiogenesis. Both transgenic animals demonstrate typical abnormalities in the formation of the vascular network. The vascular tree shows deficiencies in vessel branching with considerably fewer, simpler vessels (Sato *et al.* 1995; Patan 1998). Furthermore over-expressing Ang-1 in the transgenic animals under a keratin 14 promoter that directs gene expression only to the skin produces an increased number of highly branched vessels within the skin. (Suri *et al.* 1998). On an ultra-structural level the Tie-2 and Ang-1 knockouts both demonstrate a loss of adhesion between endothelial cells and associated pericytes and basement membranes, with the

endothelial cells rounding up and separating from adjacent structures (Suri *et al.* 1996). Taken together, these transgenic phenotypes imply a normal function for Tie-2 in the expansion of the primitive endothelial tubules to a mature capillary network either by inducing vessel branching or intussusceptive micro-vascular growth, and also for recruitment of pericytes to the newly formed tubules.

Analysis of the expression of auto phosphorylated Tie-2 in adult tissues is consistent with a dual role for Tie-2 signaling both in endothelial survival and active angiogenesis. Although Tie-2 expression is up-regulated in areas of angiogenesis, it is also present in an auto-phosphorylated state in the entire spectrum of adult quiescent vasculature (Wong *et al.* 1997).

We analyzed the possible role for the Tie-2 signaling both in angiogenesis and in endothelial survival *in vitro* using two approaches. First we analyzed the ability of recombinant Ang-1 protein to induce the migration of endothelial cells from a confluent monolayer into a collagen matrix and form tubule like structures, an assay which is analogous to the expansion of the vascular network *in vivo*. Second we eliminated Tie-2 mRNA using an antisense oligonucleotide in order to assess the functional role of Tie-2 in confluent endothelial cells, that are analogous to the quiescent endothelium *in vivo*. We report both that recombinant protein can induce endothelial cells to migrate into collagen gels and form a primitive capillary-like network and that elimination of Tie-2 mRNA and protein by the antisense treatment results in death of endothelial cells and subsequent apoptosis. These findings are consistent with a role for Tie-2 signaling both in endothelial cell survival and angiogenesis.

Materials and Methods

Cell Culture Adult Bovine Aortic Endothelial (ABAE) cells were a gift from Dr Peter Bohlen (ImClone New York) . NIH 3T3 cells were obtained from the Lombardi Cancer Center Tissue Culture Core Facility (Georgetown University Medical Center, Washington DC). Cells were maintained in IMEM (Biofluids) and 10 % FCS and 5mM Glutamine. ABAE cells were supplemented with 1ng/ml Fibroblast Growth Factor- 2 (FGF-2) (R& D systems). Cells were cultured in 37°C/5%$C0_2$ and passaged by the addition of 0.05%Trypsin/ 0.53mM EDTA (Biofluids). All experiments were performed using ABAE cells between passages 8 and 14.

Reagents Synthesis and purification of phosphorothioate oligodeoxynucleotides was performed by Genosys . Antisense (5'-GCTAAAGAATCCATGCTTCCCC-3) and sense (5'-GGGGAAGCATGG ATTCTTTAGC-3) oligonucleotides were constructed that corresponded to base pairs 318-337 of bovine Tie-2 mRNA, which spans the AUG translation initiation codon. Both sequences were compared to the NIH nucleotide database confirming the specificity of the antisense oligonucleotide to Tie-2 mRNA and also that the sense control oligonucleotide did not correspond to other known mRNA sequences

In vitro angiogenesis assay. Routine media without basic FGF, freshly prepared 1.8 % $NaHCO_3$ and type 1 rat tail collagen (Collaborative Biomedical Products) were chilled individually on ice for 1hr and then mixed in the ratio 3:1:1 by volume. The mixture (0.5 ml) was pipetted into each well of a 24 well plate and incubated at room temperature for three hours to allow the gels to solidify. At the end of this period ABAE cells were seeded in each well at density of 50,000 cells/well in 0.5ml of media containing FGF-2 (1ng/ml). The plates were incubated routinely and when the cells reached 80% confluence, each well was washed twice with PBS and then 0.5

ml of media without FGF-2 was added. After 48hrs, the media was replaced with media supplemented with the various concentrations of test compounds and the plate incubated for a further 48hrs at 37°C/5%$C0_2$ to allow tubule formation. At the end of this period the plate was chilled on ice for 15 minutes, each well was washed with cold PBS and the gels fixed with 0.5 mls of cold methanol (-20°C) for 15 minutes. Each well was washed 3 times with cold PBS then the gels covered with 0.2 mls of 50% glycerol in PBS. The next day the glycerol was removed and the gels transferred to microscope slides. Coverslips, supported by two thin pieces of wire (1mm thickness) were placed onto the gels so as to produce an entirely flat gel surface while the wire supports prevented the gel from being crushed by atmospheric pressure. The gel was surrounded by 50% glycerol, and the gap between the coverslip and slide sealed with Permount (Fischer Scientific). The following day the gels were visualized by phase contrast microscopy using a x 6.3 or x 16 objective on an inverted Zeiss microscope and images recorded using a panasonic CCD 72SX digital camera (Panasonic). Each image was analyzed using Optimas 5.2 image analysis software (Optimas Corporation). FGF-2 treated gels for transmission electron microscopy were fixed in 3% glutaraldehyde, post fixed for one hour in 1% osmium tetraoxide in cacodylate buffer and dehydrated with ethanol. Sections were imaged with a JEOL 100CX-11 transmission electron microscope.

Treatment of cells with Tie-2 sense and antisense oligonucleotides Confluent cells were harvested by trypsinization and seeded in 12 well plates at a density of 50,000 cells per well in 1 ml of culture media (IMEM /10 % FCS / 5mM glutamine supplemented with 1ng/ml FGF-2) and allowed to reach confluence (3 days). Each well was washed twice with Optimem media (Gibco BRL) with no serum or FGF-2 supplementation. The appropriate oligonucleotides were added to the optimem medium containing GC-30:DOPE lipid solution (kindly provided by Dr. Bob Brown, Genta Inc., San Diego, CA) at 1:9 ratio of oligonucleotide: lipid by weight and the cells were incubated for 4hrs at 37°C/5%$C0_2$. After incubation each well was washed twice with Optimem then incubated for a further 20 hrs with 1ml of standard culture media after which time the treatment was repeated. Cells were assessed after 48hrs from the start of the first treatment. At the end of 48 hrs the cells were washed twice with PBS then collected by incubation with 0.5ml per well of Trypsin /EDTA at 37°C which was neutralized with 1ml of culture media after all cells had detached. Aliquots from each well were counted on a Coulter Counter.

Western analysis. Cell lysates were prepared as follows. After treatment was complete, the cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 50 ug/ml approtinin, 50 ug/ml leupeptin, and 2 mM sodium orthovanadate). Total protein concentration was determined with BCA protein assay reagent (Pierce). Equal amount of proteins was subjected to SDS PAGE on an 8-16% Tris Glycine gradient gel (Novex) The proteins were electroblotted onto Hybond ECL nitrocellulose membrane (Amersham) and then blocked with 3%BSA in TBST(10mM Tris pH 7.5 150mM NaCl, 0.2% polyoxyethylene-sorbitan monolaureate (Tween-20)). Tie-2 receptor was detected with a monoclonal antibody obtained from Dr K Peters (Duke University NC) incubated with a horseradish peroxidase linked anti-murine secondary antibody then visualized by chemiluminescene (ECL Amersham). Equal loading of protein was confirmed by staining with Ponceau S (Sigma) according to the manufacturers instructions.

ISEL staining. Cells were stained for apoptotic bodies using a modification of an in situ end labeling (ISEL) technique described previously (Wijsman *et al.* 1993). ABAE cells were treated with oligonucleotides as described above, all media changes being collected and stored at 4°C so as all detached cells were collected. At the end of the treatment period adherent cells were collected by trypsinization and these cells were pooled with the previously collected media from the same treatment group. The cells were centrifuged at 1000 rpm in a bench top centrifuge and the pellet washed with ice cold PBS. This procedure was repeated after which the cells were fixed in 10% paraformaldehyde for 10 minutes. The cells were centrifuged and washed in PBS on two times and then re-suspended in a small volume of PBS, aliquots of which were placed on microscope slides. These slides were air-dried, re-hydrated with PBS for 10 minutes and then immersed in 0.3% hydrogen peroxide for 30 minutes. The slides were incubated at room temperature for 5 minutes in Buffer A (containing 0.05M Tris pH 7.5, 0.005M $MgCl_2$, 0.001% 2-mercaptoethanesulphonic acid (Sigma), 0.005% BSA, fraction V (Sigma)) and then incubated at 37°C for 1 hr with 400 µl of Buffer A per slide containing 0.2mM dCTP, dATP and dTTP (Promega), 0.02mM biotin-16-UTP (Boerhinger Mannheim) and 20 U/ml Klenow DNA polymerase (Boerhinger Mannheim). The slides were washed twice in PBS and then incubated with an avidin-biotin-horseradish peroxidase conjugate prepared in PBS (Vectastain ABC, Vector Laboratories Burlinghame CA). The slides were incubated with VIP substrate (Vector), lightly counter-stained with methyl green (Vector), dehydrated and mounted with Permount (Fisher Scientific).

Results

Recombinant Ang-1 protein induces the formation of capillary tubule like structures in collagen gels. Previous reports indicated that Ang-1 does not induce endothelial cell proliferation *in vitro*, despite inducing receptor auto-phosphorylation (Davis *et al.* 1996), and our experience with recombinant Ang-1 protein in endothelial cell mitogenesis assays confirmed this lack of mitogenic activity (data not shown). We therefore attempted to identify other phenotypic responses to Tie-2 activation by analyzing the effect of Ang-1 on the ability of confluent ABAE cells to form capillary like structures in gels of collagen. Endothelial cells when seeded on the surface of gels of basement membrane extracts, such as collagen, will normally grow until they reach confluence. At this point they can be induced to form tubule like structures by the addition of a variety of agents including phorbol esters and FGF-2 (Maciag *et al.* 1982; Feder *et al.* 1983; Montesano and Orci 1985; Montesano and Orci 1987; Goto *et al.* 1993). Figure 1A demonstrates the typical cobblestone appearance a confluent monolayer of endothelial cells cultured on collagen gels. The addition of a suitable angiogenic promoter, in this case FGF-2 (Figure 1 B) induces marked morphological changes. Endothelial cells invade into the underlying gel and form a latticework of interconnecting cellular cords. Figure 1C demonstrates the appearance of ABAE cells on collagen gels after the addition of 100ng/ml Ang-1. There is a moderately well developed network of capillary like structures that is similar to, though less extensive than, those induced by FGF-2. This assay is viewed as representative of the process of capillary sprouting and tubule formation because the endothelial cell invasion is associated with protease activity, collagen degradation, and the structures can be shown to constitute endothelial cell tubules surrounding a lumen on cross-sectional analysis (Montesano and Orci 1985 and Figure 2). In order to quantify the extent of tubule formation we utilized an image analysis

system that records the total length of the tubule like structures in a given area (Figure 2 Panel A&B). Ten images were recorded randomly from gels from each treatment group and subjected to image analysis using this system. Induction of tubule formation by Ang-1 is dose dependent (Figure 2 D). The maximum amount of tubules induced by Ang-1 (1000 ng/ml) is about 50% of that induced by FGF-2 (50ng/ml). The tubule forming effect can be entirely abrogated by the addition of an excess of soluble Tie-2 extra-cellular domain that functions effectively as a blocking antibody (Figure 1D and Figure 2E). To validate the accuracy of the image analysis system as a means of quantitating the extent of capillary-like tubule formation, we also assessed the tubule formation induced by FGF-2 and Ang-1 in Figure 2D manually. The same 10 randomly selected images per data point were ranked, in a blinded fashion, from 1 to 10 for extent of tubule formation compared with a panel of ten standard images by three independent observers. The extent of tubule formation by Ang-1 at the IC50 of 100ng/ml was within 6% of that scored by the image analysis system when normalized to the positive control (data not shown).

Elimination of Tie-2 by incorporation of a TIE-2 antisense oligonucleotide into confluent endothelial cells results in cell death. To find out if functional Tie-2 was necessary for the survival of confluent endothelial cells we eliminated Tie-2 protein by transfecting confluent endothelial cells with an antisense oligonucleotide against Tie-2 mRNA. An antisense phosphorothioate oligo-deoxynucleotide complementary to the start codon region of the Tie-2 mRNA was constructed, previous studies having demonstrated this site as likely to result in successful hybridization (Stein and Cheng 1993). This oligonucleotide was introduced into cultured ABAE cells by mixing with a lipid transfection reagent. A mixture of the lipids and the oligonucleotide at a final concentration of 150 nM oligonucleotide caused death of about 90% of the confluent ABAE cells, as determined by the number of cells that remained attached to the culture dish and the amount of total protein in the cell lysate of cells that remaining attached. Neither the lipids nor the oligonucleotide alone had any adverse effect on the cells. Additionally a corresponding sense oligonucleotide had no effect on ABAE attachment. A fibroblast cell line (NIH 3T3) that does not express Tie-2 was also not affected by the antisense oligonucleotide treatment (Figure 3B). We analyzed both cell detachment and Tie-2 protein expression in response to various concentrations of antisense oligonucleotide and could demonstrate dose-dependant cell detachment and a corresponding dose-dependant loss of Tie-2 protein expression (Figure 3C&D). Equal loading of protein was confirmed by staining total protein with ponceau S (Sigma) to visualize $\beta_1\beta_2$ and γ globulins.

Treatment with antisense oligonucleotide to TIE-2 results in apoptosis We then assessed the rate of apoptosis in ABAE cells treated by antisense or sense oligonucleotides by in situ end labeling (ISEL). ABAE cells were treated with oligonucleotide on two occasions for four hours each in serum free media, and then returned to normal media for a further 20 hrs incubation. At the end of the total 48 hr incubation period, remaining adherent cells were collected and pooled with cells that had already detached. These cells were stained by ISEL for fragmented DNA. Intact DNA does not incorporate any labeled nucleotides, whereas fragmented DNA, as occurs in apoptosis, incorporates biotinylated nucleotides. This fragmented DNA can be visualized as darkly staining discreet apoptotic bodies and these morphological appearances allow distinction between cells undergoing apoptosis and simple necrosis, where the staining is amorphous. Controls were in the form of treatment with the sense oligonucleotide, or changes of media to

serum free media that contained no oligonucleotide. Figure 4 (A&B) demonstrates the appearance of ABAE cells staining positively for fragmented DNA with numerous purple apoptotic bodies which are not seen with the sense control. Figure 4C shows results of a representative experiment in which the rate of apoptosis is increased six fold or greater over controls. The rates of apoptosis identified using this technique were very similar to those seen after 20 hours of treatment with 3μg/ml cycloheximide, a protein synthesis inhibitor that effectively induces apoptosis in endothelial cells (data not shown).

Discussion

Developmental angiogenesis is a coordinated process requiring integrated signaling through a number of ligand activated receptor tyrosine kinases that are specifically expressed on endothelial cells in a temporally coordinated manner (Dumont *et al.* 1995). The functions of the Tie-2 /Ang-1 signaling pathway in this process, as suggested by the phenotypes of the transgenic mice, include maintenance of the endothelial cell population and vascular expansion by vessel branching, intussusceptive vessel division and pericyte recruitment (Dumont *et al.* 1995; Sato *et al.* 1995; Suri *et al.* 1996; Patan 1998; Suri *et al.* 1998)

We have presented evidence at a cellular level that supports these putative roles for the Tie-2 signaling pathway both in vascular expansion and in endothelial cell survival. Our data from the collagen gel assays suggests an active role in Tie-2 signaling in tubule formation. The phenotypic analysis of both Ang-1 and Tie-2 knockout animals shows decrease in the amount and complexity of capillary branches while over-expression of Ang-1 increases the number and branching complexity of vessels, suggesting that Tie-2 signaling is necessary for the expansion of primitive tubules. *In vitro*, Ang-1 is able to induce migration of ABAE cells (Witzenbichler *et al.* 1998) and both Ang-1 and Ang-2 appear to be able to synergize with VEGF to induce angiogenesis in a mouse corneal pocket assay (Asahara *et al.* 1998). The collagen gel assay that we utilized exhibits many of the elemental endothelial cell activities during angiogenesis. The endothelial cells migrate from a confluent monolayer into a substratum and undergo proliferation, migration associated with proteolytic degradation of collagen, and ultimately organization into tubular structures that possess lumens (Maciag *et al.* 1982; Feder *et al.*1983; Montesano and Orci 1985; Montesano and Orci 1987; Goto *et al.* 1993). Soluble Ang-1 induces approximately 50 % of the tubule like structures that are induced by FGF-2. This effect can be entirely abolished by the addition of an excess of soluble Tie-2 extra-cellular domain. This evidence is in keeping with existing evidence for a dynamic role for Ang-1 in angiogenesis, particularly in the more complex morphological transitions that are involved in the expansion of an existing vascular system that may not involve mitogenesis. Such angiogenic processes may include capillary sprouting, which is known to occur under conditions where endothelial cell mitogenesis has been prevented (Sholley *et al.* 1984), and the expansion of pre-existent collateral vasculature as is seen in response to ischemia. Both of these processes can be induced by Ang-1(Koblizek *et al.* 1998; Shyu *et al.* 1998).

The hypothesis that Tie-2 signaling is a necessary endothelial cell survival factor is suggested by the phenotype of Tie-2 knockout mice, and also from the observation that Tie-2 phosphorylation results in downstream activation of protein kinase B/Akt, a protein that is central to cellular anti-apoptotic signaling pathways (Kontos *et al.* 1998). Elimination of Tie-2 protein expression in confluent endothelial cells with an anti Tie-2 antisense oligonucleotide is extremely detrimental to the cells and this effect is specific to endothelial cells, as no effect was

observed in cells that do not express Tie-2. The most striking characteristic of this treatment is the almost complete cellular detachment induced by antisense treatment. Subsequent endothelial cell apoptosis may be a direct consequence of the loss of attachment, a phenomenon referred to as "anoikis". The ultra-structural abnormalities in transgenic animals that are deficient for Ang-1 do suggest a role for Tie-2 signaling in endothelial cell attachment to adjacent cellular structures or basement membranes (Suri *et al.* 1996), but as yet the mechanism by which such a cellular effect is mediated remains unclear. Our anti sense data, where elimination of Tie-2 protein results both in endothelial cell detachment from the substratum and apoptosis, lends support to the hypothesis that this pathway is necessary for the stable association between the endothelial cell and surrounding structures.

In summary we have presented *in vitro* evidence that is highly supportive of a central physiological role for the Ang–1 /Tie-2 signaling pathway in the expansion of the endothelial cell population into a network of capillary tubules and also in the survival and stabilization of the quiescent endothelial monolayer. Further investigation will be directed toward the elucidation of Ang-1/Tie-2 signaling pathways involved in these two seemingly separate functions. This elucidation of the physiological role for the Tie-2 signaling pathway further emphasizes potential therapeutic avenues in a number of diseases manifesting altered angiogenesis, either by preventing signaling as an attempt to prevent pathological angiogenesis as seen in tumors or by augmenting signaling as an approach to ischemia (Baumgartner *et al.* 1998; Lin *et al.* 1998).

Figure Legends

Figure 1 Induction of adult bovine aortic endothelial (ABAE) cell capillary-like tubule formation by Ang-1. **Panel A,** The typical cobblestone morphology of untreated cells with cells adopting a polygonal appearance. **Panel B,** 50ng/ml FGF-2. The cells form a lattice of inter-connecting cords within the gel. **Panel C,** Tubular structures induced by 100ng/ml Ang-1. **Panel D,** Inhibition of Ang-1 induced tubule formation by 25μg/ml of soluble Tie-2 extra-cellular domain.

Figure 2 Quantification of Ang-1 induced tubule formation. **Panels A and B** illustrate the quantification of the total length of tubules formed in a gel by a computer-assisted image analysis system. **Panel C,** The total length of Ang-1induced tubule formation is dose dependent. **Panel D**, Inhibition of Ang-1 induced tubule formation by an excess of soluble Tie-2 extra-cellular domain. (Error bars = S.E.M).

Figure 3 Detachment of confluent endothelial cells caused by Tie-2 antisense oligonucleotide. **Panel A**. Effect of 150nM Tie-2 antisense on ABAE cell adhesion (AS: antisense, S: sense, L: lipid). Quantification of cell death was carried out by trypsinization and counting cells on a Coulter counter or by measuring total protein concentration of the cells that remained attached after treatment. Results are expressed as a percentage of values for untreated cells (Error bars = S.D.). Only treatment with antisense oligonucleotide in the presence of lipid resulted in cell death (* = Student's t-test $p<0.001$). **Panel B.** 300nM Tie-2 antisense had no significant effect on NIH3T3 cells while resulting in endothelial cell death. Results expressed as a percentage of sense treated control (Error bars = S.D, * = Student's t-test $p<0.001$). **Panel C**. Cell death in response to decreasing doses of antisense oligonucleotide demonstrating a dose dependent effect

(Error bars = S.D.). **Panel D.** Western blotting analysis for Tie-2 protein prepared from cells treated in Panel B confirming a dose dependent loss of Tie-2 protein that correlates to cell death.

Figure 4. Tie-2 antisense oligonucleotide treatment causes apoptosis in endothelial cells. **Panel A and B.** ISEL staining for apoptotic bodies in cells treated with antisense (A) and sense (B) oligonucleotides. Note the darkly staining purple peripheral apoptotic bodies in panel representing condensations of fragmented chromatin. **Panel C**. Quantification of apoptosis. Panel C represents the percentage of apoptotic cells as identified by ISEL staining from 1000 cells counted by a "blinded" observer. (Error bars = S.E.M)

KEY RESEARCH ACCOMPLISHMENTS:

- Angiopoietin-1 is able to induce the formation of capillary tubules by endothelial cells.
- TIE-2 is essential to the survival of endothelial cells.

REPORTABLE OUTCOMES

Manuscript: Hayes, A, Huang, WQ, Mohla, J, Yang, D, Lippman, ME, and Li, LY. Angiopoietin-1 and its receptor Tie-2 participate in the regulation and survival of endothelial cells. Microvascular Research, in press, 1999

Meeting presentation: Hayes, A, Huang, WQ, Yang, D, and Li, LY: Angiopoietin-1 Pseudomonas exotoxin fusion toxin as an anticancer therapeutic agent targeting tumor vasculature. Keystone Symposia, Taos, NM, 1999

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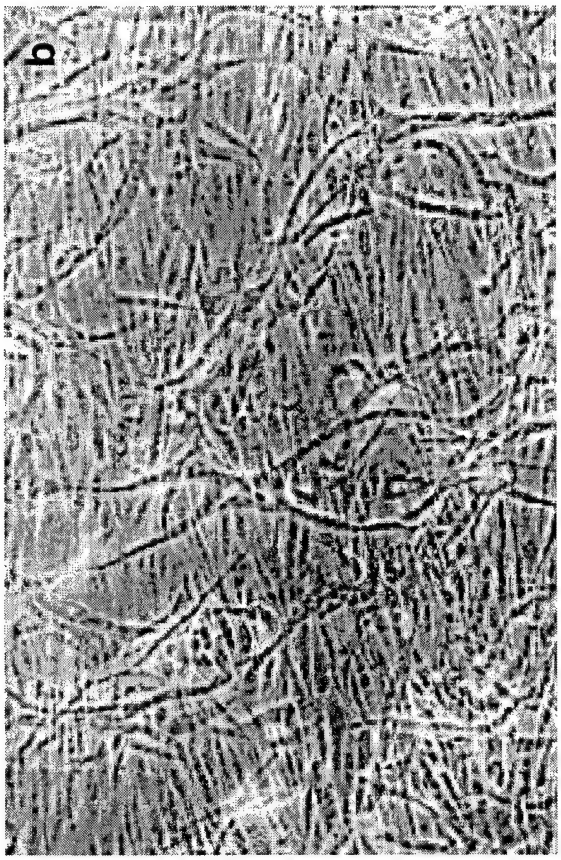
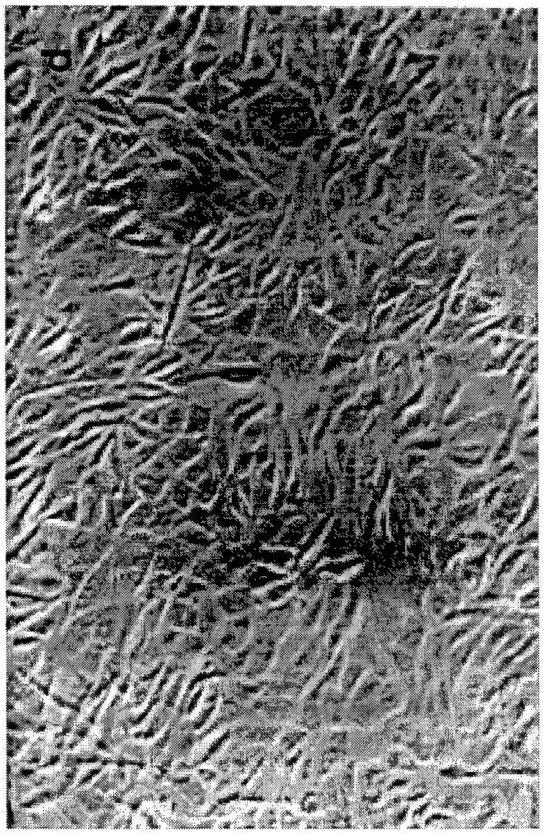
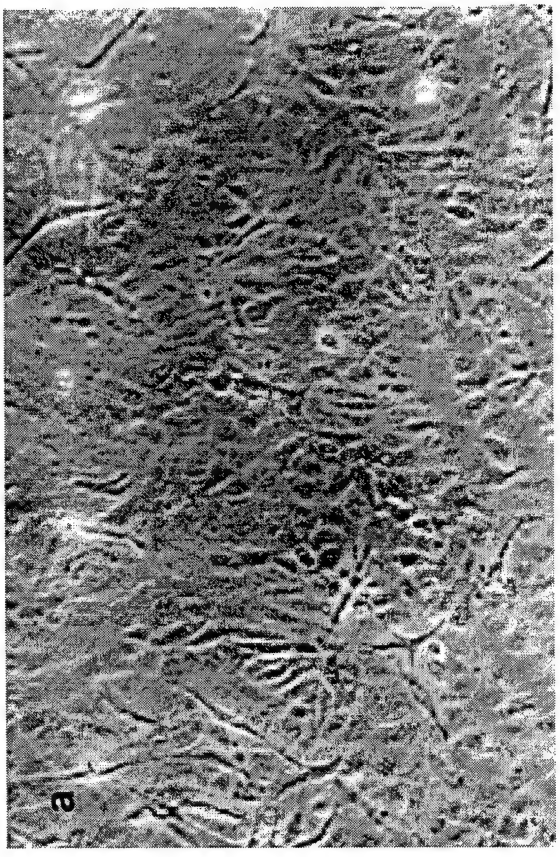
APPENDICES:

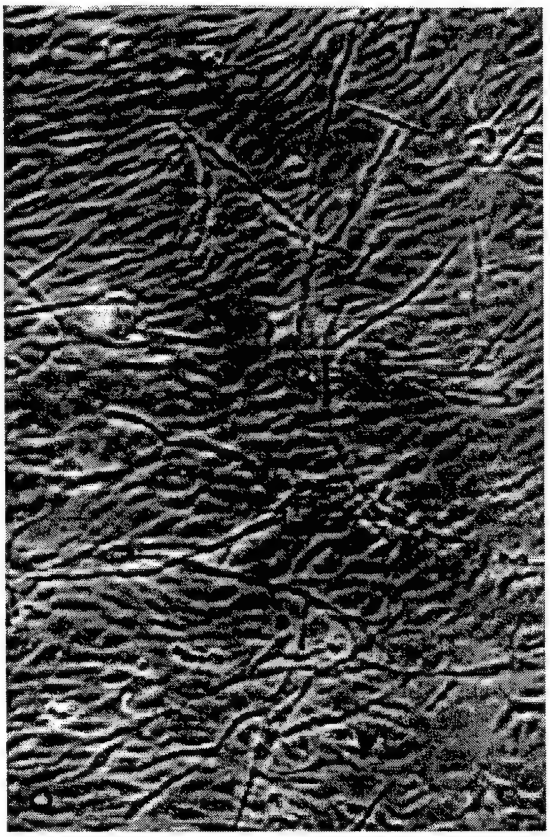
Figures 1-4

Figure 1









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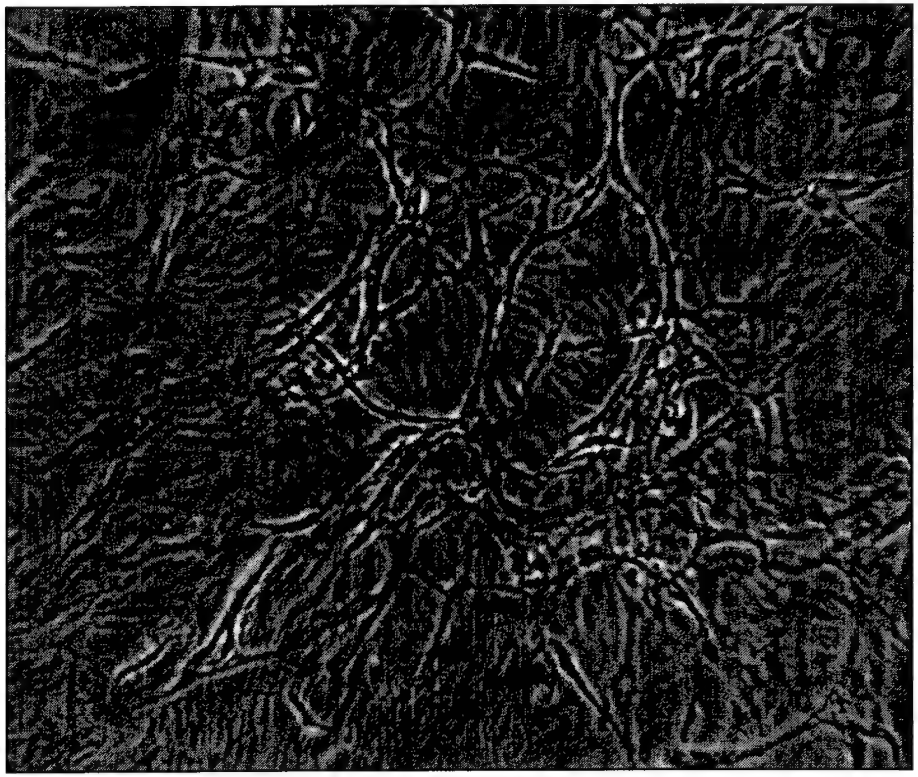

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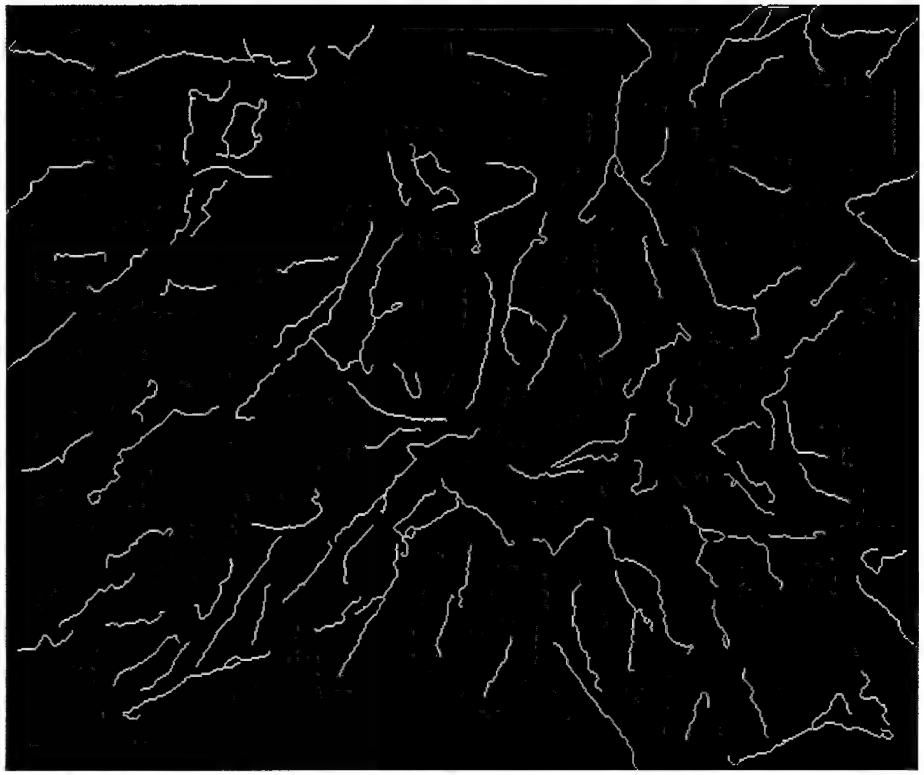


Figure 2 A & B

Figure 2C

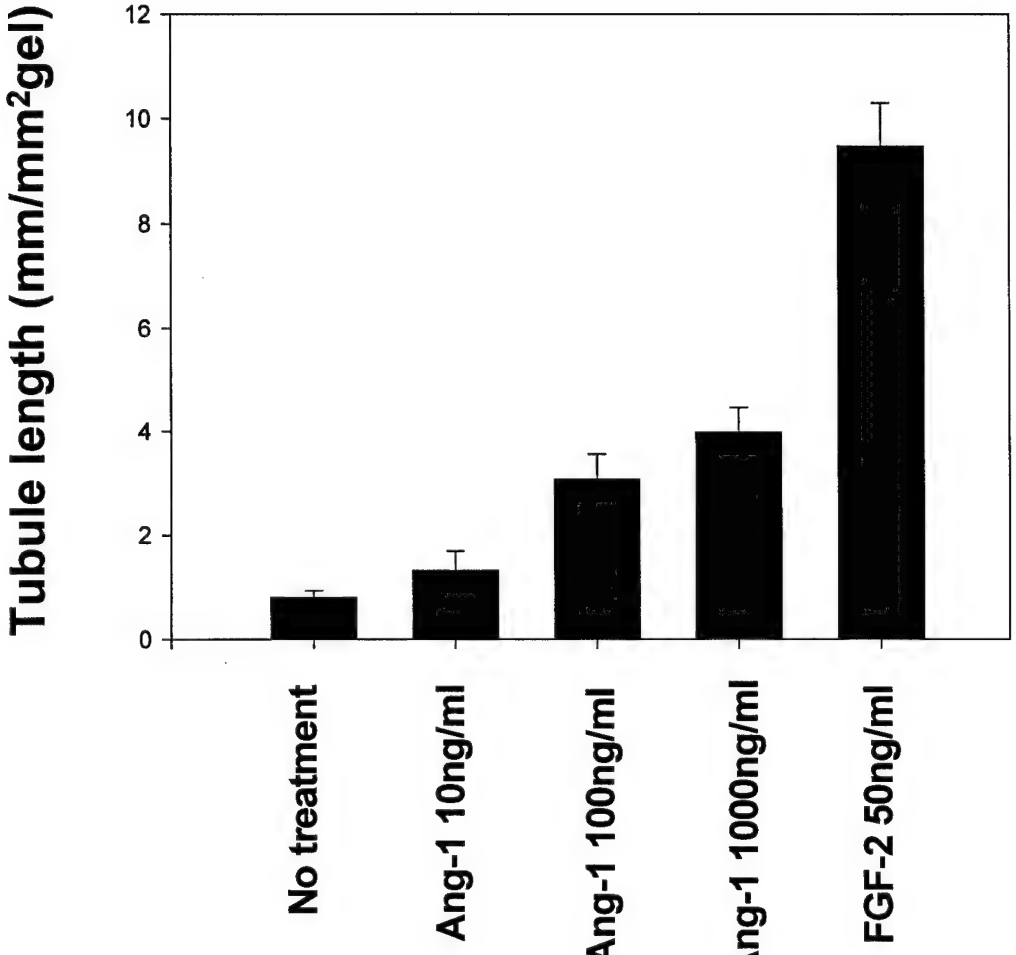

Tubule length (mm/mm²gel)
12
10
8
6
4
2
0
No treatment
Ang-1 10ng/ml
Ang-1 100ng/ml
Ang-1 1000ng/ml
FGF-2 50ng/ml

Figure 2D

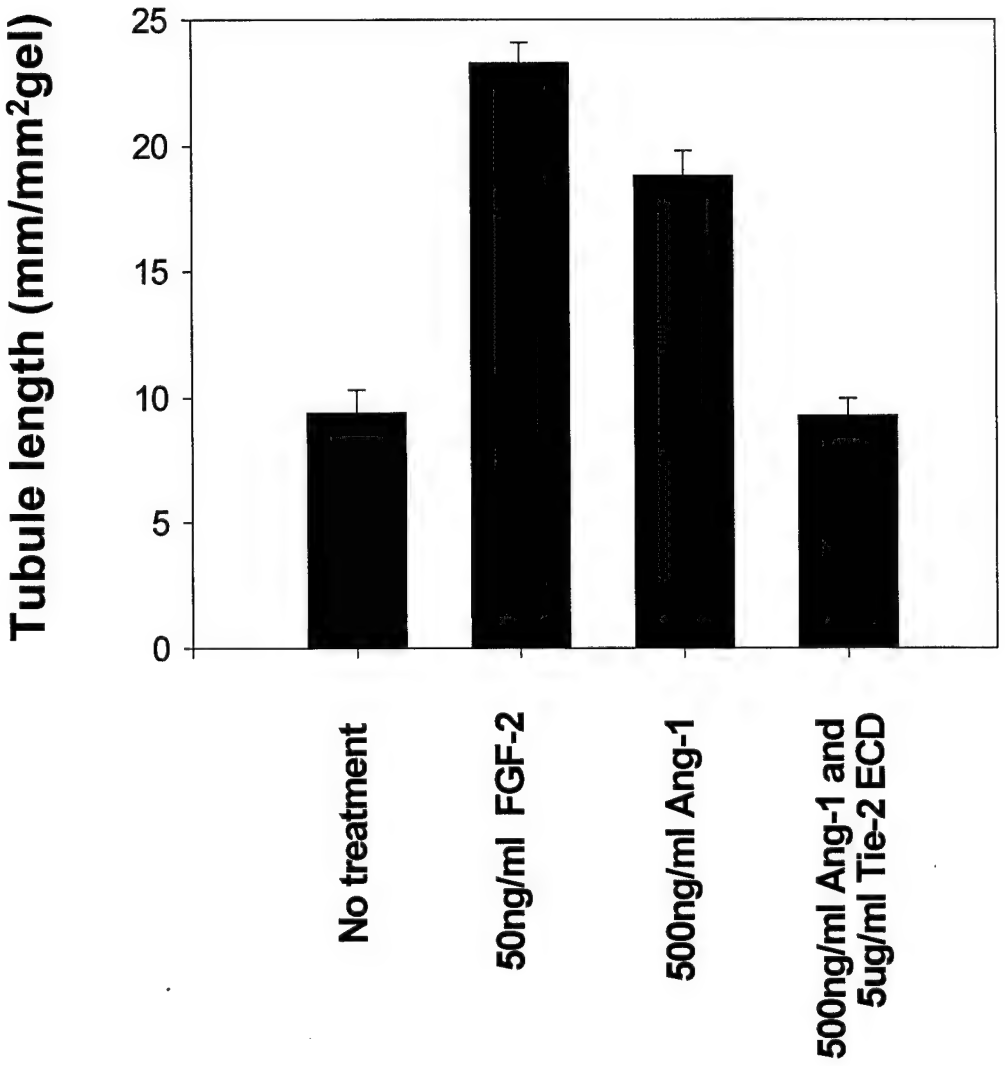

Tubule length (mm/mm²gel)
25
20
15
10
5
0
No treatment
50ng/ml FGF-2
500ng/ml Ang-1
500ng/ml Ang-1 and 5ug/ml Tie-2 ECD

Figure 3A

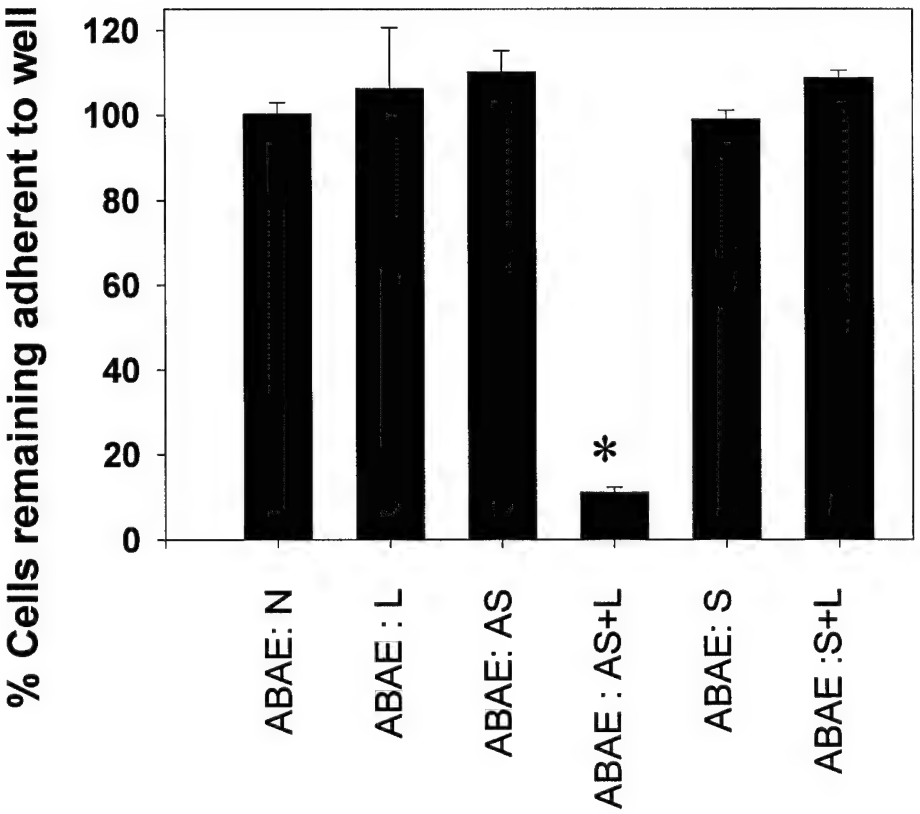

% Cells remaining adherent to well
120
100
80
60
40
20
0
*
ABAE: N
ABAE : L
ABAE: AS
ABAE : AS+L
ABAE: S
ABAE :S+L

Figure 3B

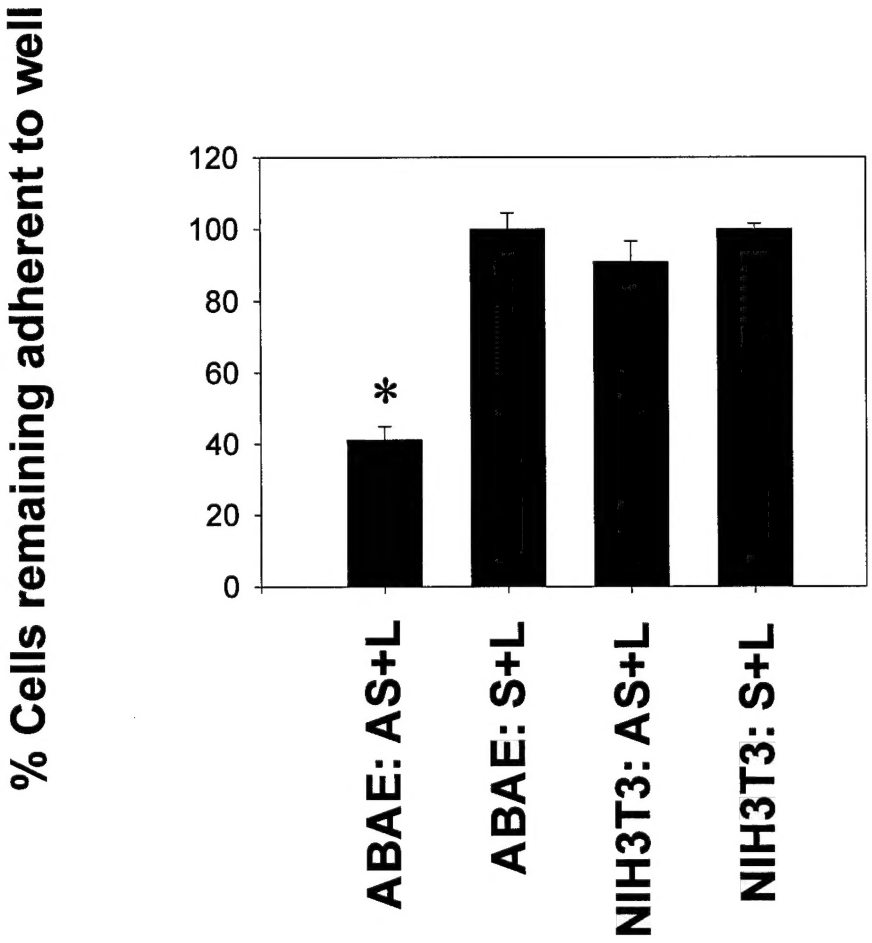
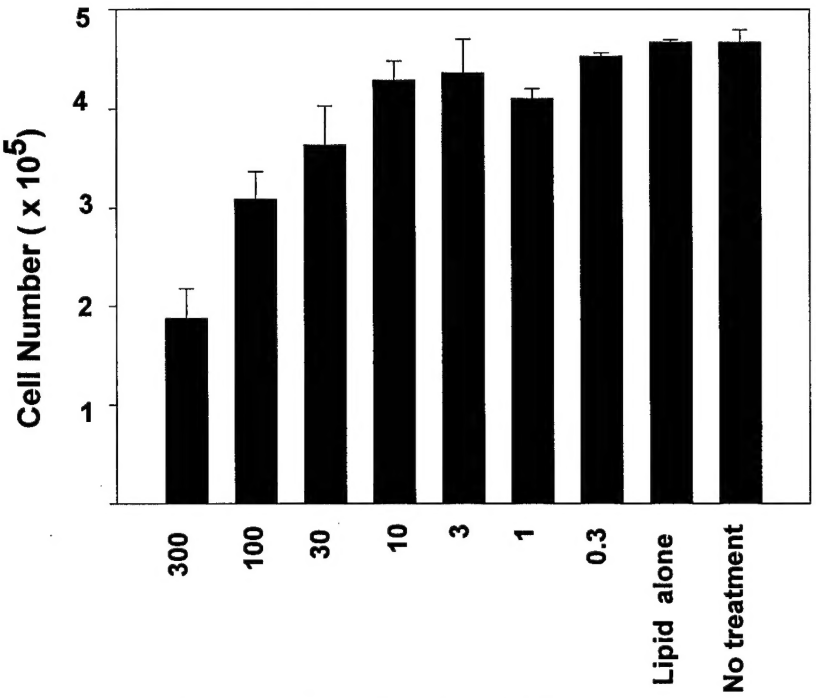
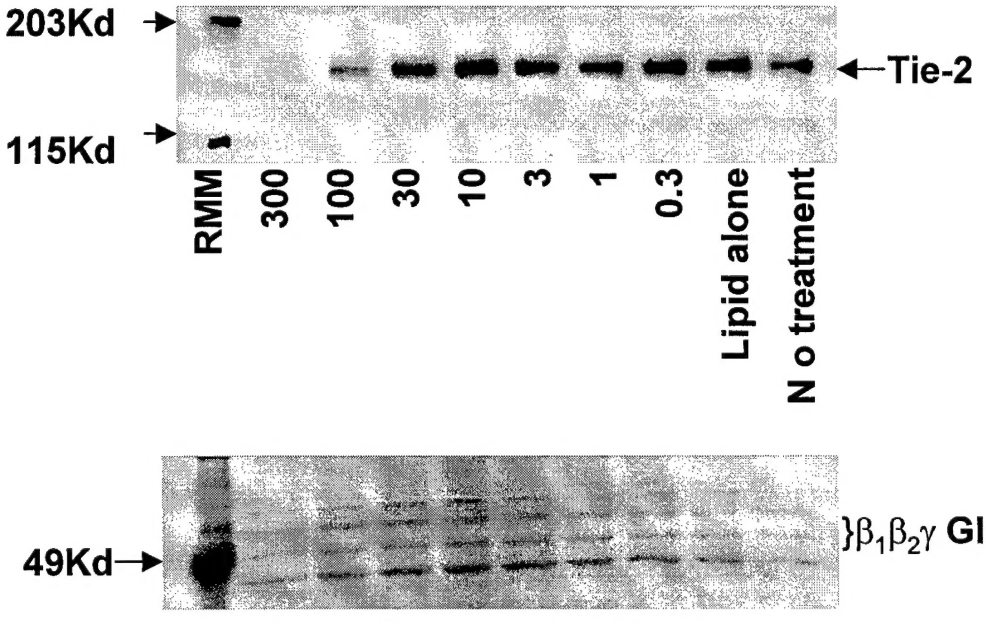

% Cells remaining adherent to well
120
100
80
60
40
20
0
*
ABAE: AS+L
ABAE: S+L
NIH3T3: AS+L
NIH3T3: S+L

Figure 3 C & D



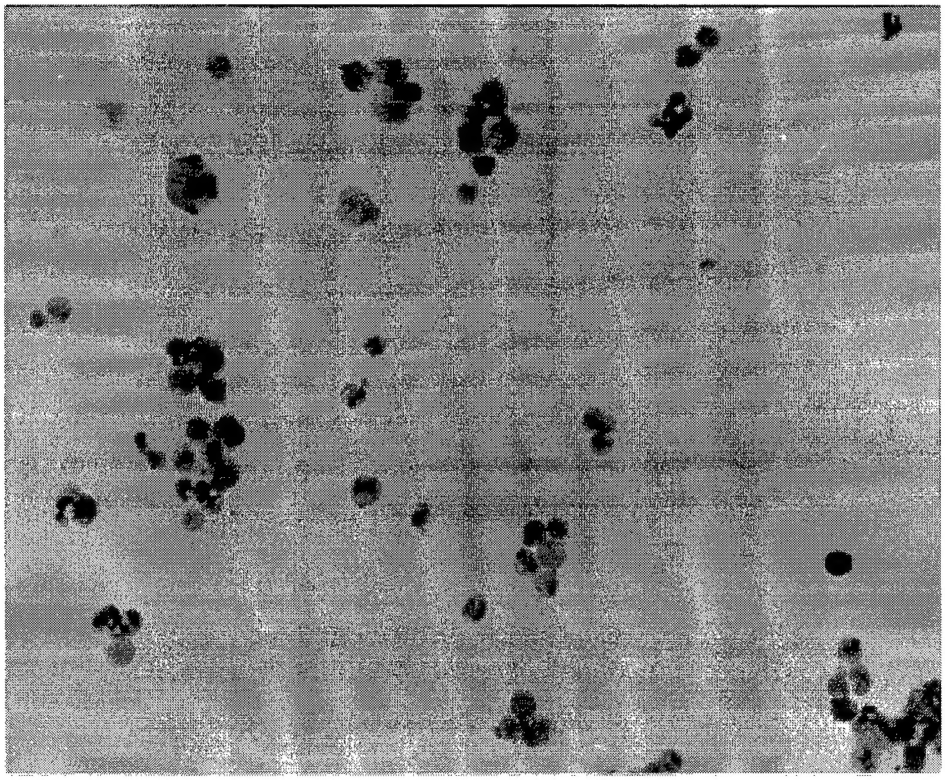
C



D

Figure 4 A & B

a



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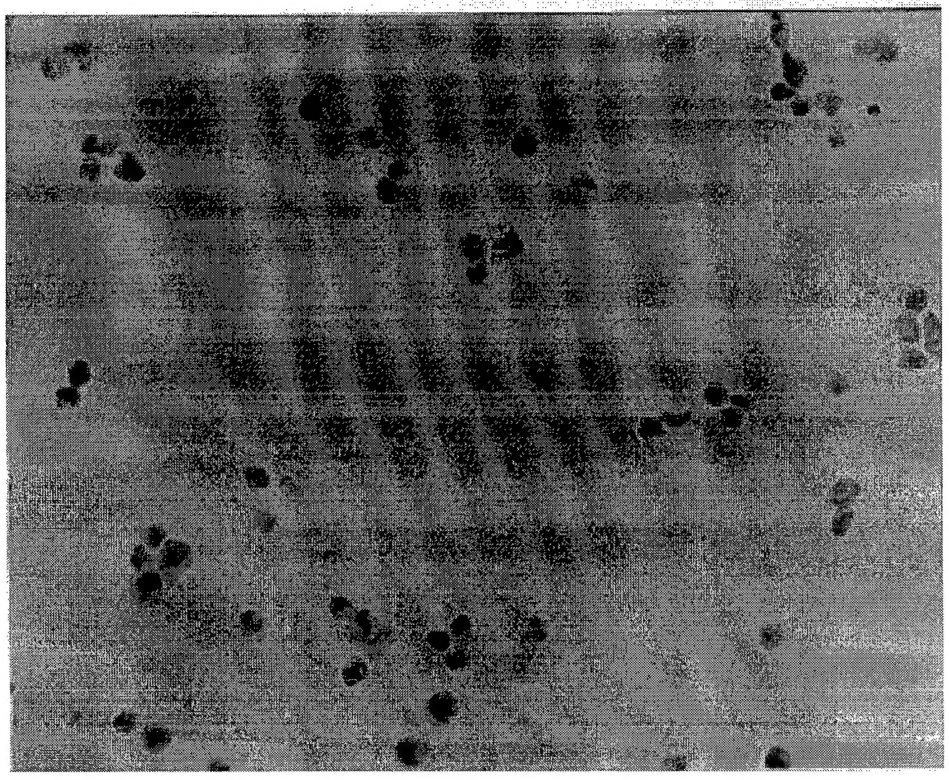
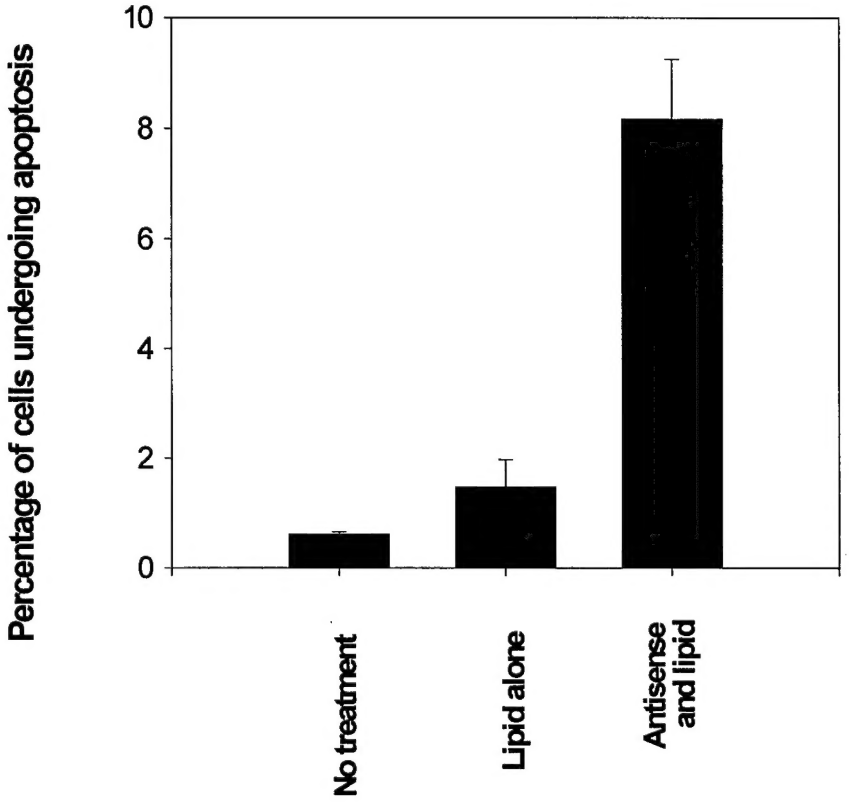


Figure 4C


Percentage of cells undergoing apoptosis
10
8
6
4
2
0
No treatment
Lipid alone
Antisense and lipid